Threshold of Detection of *Cryptosporidium* Oocysts in Human Stool Specimens: Evidence for Low Sensitivity of Current Diagnostic Methods

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To determine the minimum number of *Cryptosporidium* oocysts that can be detected in stool specimens by diagnostic procedures, stool samples seeded with known numbers of *Cryptosporidium parvum* oocysts were processed by the modified Formalin-ethyl acetate (FEA) stool concentration method. FEA concentrates were subsequently examined by both the modified cold Kinyoun acid-fast (AF) staining and fluorescein-tagged monoclonal antibody (immunofluorescence [IF]) techniques. Oocysts were more easily detected in watery diarrheal stool specimens than they were in formed stool specimens. For watery stool specimens, a 100% detection rate was accomplished at a concentration of 10,000 oocysts per g of stool by both the AF staining and IF techniques. In formed stool specimens, 100% of specimens seeded with 50,000 oocysts per gram of stool were detected by the IF technique, whereas 500,000 oocysts per g of stool were needed for a 100% detection rate by AF staining. Counting of all oocysts on IF slides indicated a mean oocyst loss ranging from 51.2 to 99.6%, depending on the stool consistency, as determined by the FEA concentration procedure. Our findings suggest that the most commonly used coprodiagnostic techniques may fail to detect cryptosporidiosis in many immunocompromised and immunocompetent individuals.

The coccidian parasite *Cryptosporidium* sp. was first reported as a human pathogen in 1976 (20, 21). As part of the evolving pandemic of infection with the human immunodeficiency virus, cryptosporidiosis gained attention as a cause of severe, life-threatening diarrhea in immunocompromised persons (6, 18, 29). More recently, the organism has been recognized as a major cause of self-limited diarrhea in immunocompetent persons (1, 6, 7, 10–15, 25, 28, 30, 33). Initially, diagnosis was based on the identification of organisms in intestinal tissue obtained by endoscopic biopsy. Following the first identification of *Cryptosporidium* oocysts in human stool samples in 1980 (31), new and varied stool concentration techniques, staining methods, and antigen detection assays for oocysts in stool have been developed and described (3, 8, 9, 17, 18, 23, 26, 29, 32, 35).

Acid-fast (AF) staining and the fluorescein-tagged monoclonal antibody (immunofluorescence [IF]) technique are among the most popular methods used in diagnostic laboratories throughout the United States. These techniques are widely believed to be sufficient for clinical purposes, since the number of oocysts excreted by symptomatic patients is thought to be high. However, the lack of an accurately defined "gold standard" has hindered any objective assessment of the sensitivity of those coprodiagnostic techniques currently in use.

To determine the efficiency of the Formalin-ethyl acetate (FEA) technique for concentrating oocysts and to define the minimum number of *Cryptosporidium* oocysts that can be detected in stool specimens by routine diagnostic procedures, we seeded stool samples with known numbers of *Cryptosporidium* oocysts and processed them by an FEA method (34) adapted from that of Ritchie (24). The concentrates were further examined for oocysts by the modified cold Kinyoun AF staining (18, 22) and IF (3, 8, 26) techniques.

MATERIALS AND METHODS

Cryptosporidium **oocysts.** Viable *C. parvum* oocysts of calf origin were stored in 5% potassium dichromate. Oocysts were recovered from fecal material by a previously described technique (5) based on the flotation method described by Sheather (27) and were fixed with 10% Formalin. The number of oocysts in the Formalin stock solution was determined by using a cell-counting chamber (Spencer Bright Line Hemacytometer; American Optical Co., Buffalo, N.Y.). All fecal specimens were seeded with 0.5 ml of dosage suspension. The number of *Cryptosporidium* oocysts in the dosage suspension was prepared by adjusting the stock solution by adding or removing Formalin.

Fecal suspensions. Volumes of 1,500 ml of two different fecal-Formalin suspensions that were free of ova and parasites were prepared. Fresh fecal material from an individual with formed stool and fresh watery diarrheal stool from a patient with AIDS were fixed with 10% Formalin in a 1:3 stool-Formalin ratio. Portions of 20 ml of stool-Formalin suspension containing 5 g of fecal material were placed in stool collection vials and individually seeded with a 0.5-ml suspension containing the number of *Cryptosporidium* oocysts necessary to yield the following concentrations: 1,000, 5,000, 10,000, 50,000, 100,000, and 500,000 oocysts per g of stool. Ten vials per oocyst concentration were prepared.

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Modified Ritchie FEA stool concentration method. The FEA stool concentration technique was performed as described previously (34). Briefly, 4 ml (formed stool) or 8 ml (watery stool) of the Formalin-fixed stool suspension was washed with water through wet gauze into 15-ml conical centrifuge tubes. The sediment, which was collected by centrifugation at 500 × g for 2 min, was resuspended in 9 ml of 10% Formalin and 3 ml of ethyl acetate and shaken vigorously for 30 s. The second centrifugation step at 500 × g for 2 min resulted in four layers (from top to bottom): ethyl acetate, plug of debris, Formalin, and sediment. The top three layers were decanted and slides were prepared by using 10-μl aliquots of the sediment.

Evaluation of the efficiency of the FEA stool concentration method. To determine whether Cryptosporidium oocysts were lost during the FEA stool concentration procedure, three parts that are routinely discarded during the procedure were retained: (i) gauze, (ii) the supernatant after the first FEA centrifugation step, and (iii) the supernatant after the second FEA centrifugation step (including the plug of debris and the Formalin layer without ethyl acetate). The gauze was washed with 15 ml of 10% Formalin, and the suspension was collected. Each part was then centrifuged separately at 500 × g for 10 min, and slides were prepared by using 10-μl aliquots of the sediment.

Staining procedures. The modified cold Kinyoun AF staining technique has been described previously (18, 22). The indirect immunofluorescence detection procedure, also described previously (3, 8, 26), was performed according to the instructions of the manufacturer (Merifluor Cryptosporidium; Meridian Diagnostics, Cincinnati, Ohio).

Examination of sediment. AF slides were examined by light microscopy at ×400 magnification, and IF slides were examined by fluorescent microscopy at ×200 (watery stool) or ×400 (formed stool) magnification. In IF slides, a ×200 magnification allows an accurate and quick detection of oocysts in very watery stools which contain little or no debris. In contrast, FEA concentrates prepared from soft and formed stools still contain considerable fecal debris. The increased background fluorescence caused by this debris requires examination at a ×400 magnification to detect low numbers of oocysts and to differentiate them from the background fluorescence.

Slides from formed stool specimens were scanned for 10 min, and the number of detected oocysts was counted. Negative slides were reviewed and scanned completely. Slides from watery stool specimens were scanned completely; the reading time averaged less than 10 min per slide. Microscopists were blinded to the number of oocysts used to seed the fecal specimens from which the slides were prepared.

Estimate of the expected minimum number of Cryptosporidium oocysts per slide. The mean number of Cryptosporidium oocysts expected on a slide if no stool concentration method was applied was estimated as follows: [no. of oocysts added per gram of stool × amount of stool (5 g)]/[volume of stool-Formalin suspension (20 ml) × volume of stool-Formalin suspension on slide (0.01 ml)].

Assuming a Poisson distribution of oocysts in the stool-Formalin suspension, the 95% confidence interval for the number of oocysts one would expect to find in a 0.01-ml sample of suspension would approximate ±2 standard deviations around the mean for mean oocyst counts of ≥15 (2).

TABLE 1. Comparison of AF staining and IF techniques for detecting Cryptosporidium oocysts in seeded watery diarrheal and formed stool specimens

No. of oocysts added per g of stool	No. of specimens positive/no. examined			
	Watery stool		Formed stool	
	AF staining	IF technique	AF staining	IF technique
1,000	0/10	0/10	ND[a]	ND
5,000	6/10	9/10	ND	2/10
10,000	10/10	10/10	0/10	6/10
50,000	10/10	10/10	0/10	10/10
100,000	ND	ND	6/10	10/10
500,000	ND	ND	10/10	ND

[a] ND, not done.

RESULTS

The threshold of detection of Cryptosporidium oocysts was different in formed and watery diarrheal stool specimens. The IF detection procedure was more sensitive than the AF staining technique for both types of specimens (Table 1). For watery stool specimens, oocysts were detected in 90% of specimens containing 5,000 oocysts per g of stool by the IF technique, whereas oocysts were detected in 60% of specimens by the AF technique. Both IF and AF techniques, however, detected all specimens seeded with 10,000 oocysts per g of stool. In formed stool specimens, the IF method detected 100% of specimens seeded with 50,000 oocysts, while 500,000 oocysts per g of stool were required for 100% detection by AF staining.

Comparison of the estimated number of oocysts expected on a slide if no concentration method was applied with the number of oocysts detected by the FEA stool concentration procedure revealed that the procedure resulted in oocyst loss rather than oocyst concentration (Table 2). For watery stool specimens, the estimated mean oocyst loss determined by the FEA stool concentration procedure was between 51.2% (IF technique, 10,000 oocysts per g of stool) and 91.2% (AF staining, 5,000 oocysts per g of stool), and for formed stool specimens, the estimated mean oocyst loss was between 93.2% (IF technique, 50,000 oocysts per g of stool) and 99.6% (AF staining, 100,000 oocysts per g of stool). Nevertheless, oocysts were more easily observed and counted in FEA-processed stool specimens than in direct smears made from the unconcentrated stool-Formalin suspension; mean oocyst counts from 10 experiments (IF technique, 100,000 oocysts per g of stool, formed stool) were seven times higher in FEA-processed specimens than in slides prepared directly from the stool-Formalin suspension.

Analyzing different steps of the FEA stool concentration technique (Table 3), we detected high numbers of Cryptosporidium oocysts in the discarded elements of the FEA procedure, i.e., in the gauze that was used for stool filtration and in the supernatants from both the first and second centrifugation steps.

DISCUSSION

By examining human fecal material seeded with known numbers of C. parvum oocysts, we sought to determine the sensitivity of coprodiagnostic tests commonly used to detect Cryptosporidium oocysts. No "gold standard" for detection of Cryptosporidium oocysts in human stool specimens has yet been established (32).

TABLE 2. Comparison between expected and observed detection of *Cryptosporidium* oocysts

No. of oocysts added per g of stool	Expected no. of oocysts on slide (± 2 SD)[a]	Mean no. (range) of detected oocysts[b]			
		Watery stool		Formed stool	
		AF staining	IF technique	AF staining	IF technique
1,000	2.5	0	0	ND	ND
5,000	12.5	1.1 (0–3)	2.9 (0–6)	ND	0.3 (0–2)
10,000	25 ($\pm$10)	7.9 (1–16)	12.2 (4–16)	0	1.2 (0–3)
50,000	125 ($\pm$22)	+++[c]	+++	0	8.5 (5–14)
100,000	250 ($\pm$32)	ND	ND	0.9 (0–3)	16.7 (11–26)
500,000	1,250 ($\pm$71)	ND	ND	27.5 (18–49)	ND

[a] Expected number of oocysts on slide without FEA concentration. For calculation, see text. SD, standard deviation.
[b] There were 10 slides for each oocyst dilution and each staining technique. Slides prepared from watery diarrheal stool specimens were scanned completely; slides from formed stool specimens were scanned for 10 min. Negative slides were reviewed and scanned completely. ND, not done.
[c] +++, 10 of 10 specimens were positive per number examined. The number of detected oocysts was not determined.

We found the minimum number of oocysts in human stool specimens that can be detected by the FEA stool concentration technique and the IF staining procedure to be unexpectedly high, 5,000 to 10,000 oocysts per g of stool in watery stool specimens and 10,000 to 50,000 oocysts per g of stool in formed stool. The yield by the AF staining technique was significantly worse than that by the IF technique, mainly in formed stool specimens. Our results may represent an oocyst detection rate based on extremes of stool consistency. The diarrheal stool we used was very watery and almost devoid of fecal debris. Less watery stools may contain significantly more fecal debris, making oocyst detection more difficult.

Our results are consistent with those of others (3, 8, 26) who have shown that the IF detection procedure is more sensitive than the AF staining technique. The IF technique offers the advantage of a readily identifiable apple green fluorescence. Nevertheless, *Cryptosporidium* oocysts may still be difficult to detect, especially in stool samples containing a low oocyst concentration or excess fecal debris, which causes increased background fluorescence.

TABLE 3. Evidence for loss of *Cryptosporidium* oocysts during FEA stool concentration procedure

Parts examined by FEA concentration technique	No. of detected oocysts in each of the following stool samples[a]:			
	1	2	3	4
Parts discarded from FEA technique				
Gauze	47	275	168	64
Supernatant 1[b]	93	235	354	79
Supernatant 2[c]	27	209	136	107
Total	157	719	658	250
FEA concentrate for examination, 0.01 ml of sediment	106	595	535	134

[a] Number of detected oocysts in 50 microscopic fields by using ×400 magnification. The IF detection procedure was used. Sample 1 was seeded with 1,000,000 and samples 2 and 3 were seeded with 5,000,000 *Cryptosporidium* oocysts per g of stool. Sample 4 was a stool specimen from an immunocompetent child with symptomatic cryptosporidiosis. Five grams of the child's stool specimen was fixed in 15 ml of Formalin and was processed by the FEA concentration technique as described in the text.
[b] Supernatant after first centrifugation step.
[c] Plug debris and supernatant after second centrifugation step.

Oocyst detection procedures require both sensitive and specific staining techniques and effective methods for concentrating oocysts in stool specimens. Our findings suggest a significant oocyst loss by the FEA concentration procedure. The detection of oocysts in the gauze used for stool filtration and in the supernatants obtained from the first and second centrifugation steps implies that modifications of the FEA concentration procedure may be required to prevent such large oocyst losses.

Our data raise questions about the widely held belief that these diagnostic techniques are sufficient to meet the needs of clinicians. This belief is based on the assumption that all symptomatic patients excrete such large numbers of oocysts that laboratory detection is assured. However, no available data support this assumption. The degree of oocyst shedding by patients suffering from cryptosporidiosis is not known. The low sensitivity of coprodiagnostic procedures might be responsible for underdiagnosis in both immunocompromised and immunocompetent individuals. Chronic diarrhea of unknown origin in patients with AIDS is a well-known diagnostic problem that might frequently be caused by an undetected *Cryptosporidium* sp. In a recent prospective analysis of jejunal biopsies in patients with AIDS, *Cryptosporidium* was present in 5 of 43 (11.6%) patients whose stool examinations were negative (16).

The high threshold necessary for oocyst detection in formed or semiformed stool specimens by coprodiagnosis may also limit early diagnosis. As therapeutic agents become available to treat cryptosporidiosis, early detection could allow for earlier treatment and possible prevention of the fulminant, life-threatening diarrhea often seen in immunocompromised patients. Furthermore, the low sensitivity of current diagnostic methods will certainly affect epidemiologic investigations that rely on detecting asymptomatic or early infection in order to assess risk factors and modes of transmission for *Cryptosporidium* spp. Indeed, a high rate of asymptomatic carriage (12.7%) has been documented in one group of immunocompetent patients undergoing endoscopy (25).

Our data indicate that new or modified coprodiagnostic techniques for *Cryptosporidium* spp. are needed. However, our preliminary attempts to improve this procedure by using plastic screens instead of gauze, adding surfactants, varying centrifugation times, modifying the relative centrifugal force, or increasing the starting volume of stool to be processed by the FEA concentration technique have been unsuccessful. Increasing the centrifugation time and increas-

ing the relative centrifugal force in processing stool-Formalin suspensions of formed stool samples produced a lower oocyst detection rate (data not shown), possibly because stool particles were compacted, and oocysts were thus bound or obscured. Although more efficient oocyst detection in watery compared with that in formed stool specimens suggests that a separation of parasites from stool debris is a crucial step in coprodiagnostic procedures, available data do not support the superiority of techniques such as sugar flotation for detection of Cryptosporidium spp. (19). Moreover, the sugar flotation technique is more cumbersome to perform, and the presence of the sugar solution of Sheather (27) inhibits staining procedures (4). Although additional washing steps after flotation can be used to remove excess sugar solution and render oocysts stainable, such washing results in a considerable loss of oocysts and an unacceptable decrease in test sensitivity (data not shown). Stool antigen detection techniques may eventually provide improved coprodiagnosis, but recent reports did not show significantly improved sensitivity (17, 32).

Hence, until better coprodiagnostic techniques are developed, both clinicians and epidemiologists need to be aware of the limitations of currently available techniques. Efforts to improve these techniques are under way.

ACKNOWLEDGMENTS

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